REMARKS

Claims 1-59 are pending, and claims 1-4, 6-14, 22-24, 26-39, and 48-59 are currently under consideration. Applicants respectfully request reconsideration in view of the following remarks. Issues raised by the Examiner will be addressed below in the order they appear in the prior Office Action. Applicants thank the Examiner for courtesies extended during a telephonic interview conducted on June 18, 2003.

1. Applicants note with appreciation that the amendment filed November 18, 2002 has been entered in full. Applicants note that claims 5, 15-21, 25 and 40-47 have been withdrawn from consideration as directed to a nonelected invention. Applicants will cancel nonelected claims upon indication of allowable subject matter.

2-3. Claims 1-4, 6, 7, 10-14, 22-24, 26, 28-39, 48, and 50-59 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for allegedly failing to particularly point out and distinctly claim the subject matter that Applicants regard as the invention. Applicants traverse this rejection and maintain that the rejection is moot in light of the amended claims.

Applicants contend that recitation of the phrase "said elastin-based composition comprising a polypeptide having an amino acid sequence at least 80% identical to SEQ ID No. 2, SEQ ID No. 3, or a peptide fragment thereof including at least one hexameric sequence represented by SEQ ID No. 1" is clear, and that one of skill in the art can readily recognize the metes and bounds of the claimed subject matter. Nevertheless, to expedite prosecution of claims directed to commercially relevant subject matter, Applicants have amended the claims to improve their clarity. Applicants' amendments are not in acquiescence to the rejection, and Applicants reserve the right to prosecute claims of similar or differing scope. Reconsideration and withdrawal of this rejection are respectfully requested.

4-5. Claims 1-4, 6, 7, 10-14, 22-24, 26, 28-39, 48, and 50-59 are rejected under 35 U.S.C. 112, first paragraph, as allegedly containing subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor had possession of the claimed invention. Applicants traverse this rejection and maintain that the rejection is moot in light of the amended claims.

In the prior Office Action, the Examiner alleged that certain terms recited in the previously pending claims constitute new matter. Specifically, the Examiner objected to recitation of the term "trachea" and also objected to the reference of polypeptides related, by a particular percent identity, to SEQ ID No. 1 or SEQ ID No. 2. Applicants respectfully disagree with the Examiner's determination that these claim amendments include new matter, and contend that the specification provides ample inherent support for recitation of these terms (see, for example, page 18, lines 4-9; page 20, lines 1-7).

The Examiner appears to be basing this rejection on an alleged lack of *in haec verba* support in the specification for the above referenced phrases. However, in accordance with MPEP 2163, the specification need not provide *in hiis verbis* support for the claimed subject matter. All that is required is that "newly added claim limitations must be supported in the specification through express, implicit, or inherent disclosure." (MPEP 2163). Applicants contend that the specification provides ample implied and inherent support for the claimed subject matter, and accordingly the claims satisfy all of the requirements under 35 U.S.C. 112, first paragraph.

Nevertheless, to expedite prosecution of claims directed to commercially relevant subject matter, Applicants have amended the claims to more particularly point out certain embodiments of Applicants' invention. Applicants' amendments are not in acquiescence to the rejection, and Applicants reserve the right to prosecute claims of similar or differing scope. Reconsideration and withdrawal of this rejection is respectfully requested.

6. Claims 1-4, 6-14, 22-24, 26-39, and 48-59 are rejected under 35 U.S.C. 112, first paragraph, as allegedly failing to enable one of skill in the art to make or use the claimed invention. Applicants traverse this rejection and maintain that the rejection is moot in light of the amended claims.

The basis of the rejection appears to be three-fold. First, the Examiner alleges that the specification fails to enable one of skill in the art to practice the claimed invention based on any route of administration. Second, the Examiner alleges that the specification fails to enable one of skill in the art to practice the invention based on administration of polypeptides comprising variant amino acid sequences. Third, the Examiner alleges that the specification fails to enable one of skill in the art to practice the invention based on administration of a polypeptide

comprising one repeat of the hexameric sequence represented in SEQ ID No. 1. Applicants contend that the specification provides a broadly enabling disclosure, and that each of the issues raised in the previous Office Action fail to undermine the patentability of the claimed invention.

With regard to the first grounds of rejection, Applicants contend that the specification is broadly enabling for pharmaceutical preparations of elastin-based compositions for administration to target sites in vivo. Furthermore, Applicants contend that any concerns that the Examiner may have had regarding the enablement of the claimed invention should have been obviated by Applicants' previous amendments to the claims that particularly pointed out that the claimed compositions are **formulated in association with a biocompatible support** and **delivered to a target site**. Accordingly, contrary to the Examiner's contention, the claims are directed to particular routes of administration.

While maintaining the rejection in the face of these amendments, the Examiner has offered no objective evidence to support the rejection. In accordance with MPEP 2164.04, "the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention....A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, unless there is a reason to doubt the objective truth of the statements contained thereon which must be relied on for enabling support." (MPEP 2164.04; *In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993)).

Applicants contend that the claims are enabled throughout their scope, and respectfully submit that the Examiner has provided no objective evidence to undermine the patentability of the claimed subject matter. Reconsideration and withdrawal of this rejection are respectfully requested.

With regard to the second grounds of rejection, Applicants contend that the specification is broadly enabling for compositions comprising variant tropoelastin sequences, as well as methods of using such variant tropoelastin sequences. MPEP 2164.01 outlines the standard to be used when assessing whether the claimed subject matter is enabled throughout its scope. The test for enablement is "whether the disclosure, when filed, contained sufficient information regarding the subject matter of the claims as to enable one skilled in the pertinent art to make and

use the claimed invention." The MPEP further reminds us that the test of enablement does not require that one of skill in the art can practice the claimed invention without any experimentation. All that is required is that one of skill in the art can practice the claimed invention without undue experimentation.

Briefly, the specification provides extensive guidance regarding the making and testing of various elastin-based polypeptides and fragments whose sequences vary from, for example, SEQ ID No. 3 (page 17, line 14-page 20, line 23; page 22, lines 5-12; page 18, lines 4-9; page 20, lines 1-7). The guidance provided in the specification includes the disclosure of exemplary functional attributes that the claimed elastin-based compositions must possess, as well as a variety of in vitro and in vivo assays that can be used by one of skill in the art to readily evaluate whether a particular elastin-based composition does in fact possess the required functional activity (page 17, lines 14-25). Accordingly, based on the specification, one of skill in the art can readily make elastin-based compositions possessing a variant sequence, and test those polypeptides to identify those that possess the desired functional attributes (i.e., the bioactivity of naturally occurring sequence).

The Examiner points out that variations in amino acid sequence can sometimes have an impact on the function of a protein, and alleges that this possibility undermines the enablement of the claimed subject matter. However, Applicants respectfully note that the reference relied upon by the Examiner regarding the effect on protein function of changes in amino acid sequence was published substantially prior to the filing of the present application. Since that time, there has been a veritable explosion in the art of combinatorial chemistry which readily allows the making and testing of polypeptide variants without undue experimentation. Thus, even if one agrees that small differences in polypeptide sequence can affect the function of a protein or peptide, this point is immaterial in assessing the enablement of the claimed subject matter. Rather, the important consideration in determining whether Applicants have enabled the use of polypeptide variants in the subject methods is whether one of skill in the art could readily make and test polypeptide variants using the teachings of the specification and the state of the art, without undue experimentation, in order to select variants for use in the subject methods. Applicants contend that this burden has been met. Enablement is assessed in terms of the level of skill in the art at the time of filing. Accordingly, as technical advances in a particular field

occur, the amount of experimentation allowable without constituting undue experimentation increases. Such is the case here.

MPEP 2164.06 and the courts have clearly articulated the standard for evaluating whether the level of experimentation necessary to practice the claimed invention is permissible. "[A] considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." (*In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988); *In re Angstadt*, 537 F.2d 489, 502-04, 190 USPQ 214, 217-19 (CCPA 1976)).

Applicants contend that in light of the extensive guidance provided in the specification, and in light of the high level of skill in the art, one of skill in the art could readily make and test polypeptide variants to identify variants for use in the claimed invention without undue experimentation. Accordingly, Applicants respectfully request reconsideration and withdrawal of this rejection.

With regard to the third grounds of rejection, Applicants contend that the specification is broadly enabling for compositions comprising a single hexameric sequence represented in SEQ ID No. 1, as well as methods of using such compositions. In maintaining this rejection, the Examiner appears to contend that simply because Applicants did not present a specific example in which an elastin-based composition comprising a single hexameric sequence represented in SEQ ID No. 1 was used to influence smooth muscle cell behavior, Applicants have not enabled the use of such elastin-based compositions. However, this reasoning is contrary to the guidance provided by the MPEP and by case law.

In accordance with MPEP 2164.02, "[a]n example may be 'working' or 'prophetic.' A working example is based on work actually performed. A prophetic example describes an embodiment of the invention based on predicted results rather than work actually conducted or results actually achieved.....The specification need not contain an example if the invention is otherwise disclosed in such manner that one skilled in the art will be able to practice it without undue experimentation." (*In re Borkowski*, 422 F.2d 904, 908, 164 USPQ 642, 645 (CCPA 1970)).

Additionally, Applicants point out that, as of the time of filing, elastin-based compositions comprising a single hexameric sequence represented in SEQ ID No. 1 were known

to have functional activity in certain in vitro systems. Applicants direct the Examiner's attention to a publication by Senior et al. which was provided to the Examiner in a previously submitted Information Disclosure Statement (enclosed herewith as Exhibit A). The teachings of Senior et al. demonstrate that an elastin peptide having the amino acid sequence VGVAPG has chemotactic activity in fibroblasts and monocytes. However, Senior et al. is silent as to the activity of such a peptide in smooth muscle cells. Applicants provide extensive evidence concerning the activity of tropoelastin and tropoelastin fragments comprising 7-repeats of VGVAPG in smooth muscle cells. In light of Applicants' demonstration of a particular activity for tropoelastin and tropoelastin fragments in smooth muscle cells, and in light of the knowledge in the art that demonstrated that a single hexamer of VGVAPG can have functional activity, Applicants contend that there is no reasonable basis to doubt the enablement of the presently claimed subject matter.

Applicants contend that the specification and the state of the art at the time of filing support the enablement of claims directed to the use of a single repeat of the hexameric sequence represented in SEQ ID No. 1 (VGVAPG). In further support of Applicants' position, Applicants submit herewith the declaration of Dean Li under 37 CFR 1.132. The declaration summarizes experiments demonstrating that an elastin peptide with a sequence identical to SEQ ID No. 1 can influence smooth muscle cell behavior in a manner similar to that of tropoelastin. Briefly, Exhibit 1 shows that an elastin peptide identical to SEQ ID NO: 1 induced actin polymerization in vascular smooth muscle cells derived from elastin -/- mice in a dose dependent, temporally sensitive manner. Exhibit 2 shows that an elastin peptide identical to SEQ ID NO: 1 promoted vascular smooth muscle cell chemotaxis as evaluated using a modified Boyden-chamber assay. The declaration of Dean Li demonstrates that, as taught in the specification as filed, an elastin-based composition comprising a single repeat of the hexameric sequence represented in SEQ ID No. 1 **does** modulate smooth muscle cell behavior in a manner similar to that of recombinant tropoelastin. This post-filing evidence further supports Applicants' contention that the teachings of the specification broadly enable the practice of the claimed invention.

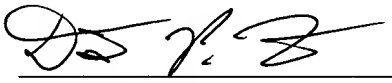
In accordance with MPEP 2164.05, when making a determination as to the enablement provided for the claimed invention, the evidence must be considered as a whole. Furthermore, "the evidence provided by the applicant need not be conclusive but merely convincing to one skilled in the art." (MPEP 2164.05). Applicants contend that this burden has been satisfied.

Applicants contend that the specification is broadly enabling for a range of elastin compositions, as well as for numerous routes of administration of such elastin compositions. Nevertheless, to expedite prosecution of claims directed to commercially relevant subject matter, Applicants have amended the claims to more particularly point out certain embodiments of Applicants' invention. Applicants' amendments are not in acquiescence to the rejection, and Applicants reserve the right to prosecute claims of similar or differing scope. In light of Applicants' amendments and arguments of record, reconsideration and withdrawal of this rejection are respectfully requested.

CONCLUSION

In view of the foregoing amendments and remarks, Applicants submit that the pending claims are in condition for allowance. Early and favorable reconsideration is respectfully solicited. The Examiner may address any questions raised by this submission to the undersigned at 617-951-7000. Should an extension of time be required, Applicants hereby petition for same and request that the extension fee and any other fee required for timely consideration of this submission be charged to **Deposit Account No. 18-1945.**

Respectfully Submitted,



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Val-Gly-Val-Ala-Pro-Gly, a Repeating Peptide in Elastin, Is Chemotactic for Fibroblasts and Monocytes

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ABSTRACT Recent studies have demonstrated that tropoelastin and elastin-derived peptides are chemotactic for fibroblasts and monocytes. To identify the chemotactic sites on elastin, we examined the chemotactic activity of Val-Gly-Val-Ala-Pro-Gly (VGVAPG), a repeating peptide in tropoelastin. We observed that VGVAPG was chemotactic for fibroblasts and monocytes, with optimal activity at $\sim 10^{-8}$ M, and that the chemotactic activity of VGVAPG was substantial (half or greater) relative to the maximum responses to other chemotactic factors such as platelet-derived growth factor for fibroblasts and formyl-methionyl-leucyl-phenylalanine for monocytes. The possibility that at least part of the chemotactic activity in tropoelastin and elastin peptides is contained in VGVAPG sequences was supported by the following: (a) polyclonal antibody to bovine elastin selectively blocked the fibroblast and monocyte chemotactic activity of both elastin-derived peptides and VGVAPG; (b) monocyte chemotaxis to VGVAPG was selectively blocked by preexposing the cells to elastin peptides; and (c) undifferentiated (nonelastin producing) bovine ligament fibroblasts, capable of chemotaxis to platelet-derived growth factor, did not show chemotactic responsiveness to either VGVAPG or elastin peptides until after matrix-induced differentiation and the onset of elastin synthesis. These studies suggest that small synthetic peptides may be able to reproduce the chemotactic activity associated with elastin-derived peptides and tropoelastin.

Chemotactic activity has been associated with several components of the extracellular matrix including collagen (18), fibronectin (1, 5, 17), laminin (27), peptides of insoluble elastin (6, 22, 23), and tropoelastin (23). The sites of chemotactic activity on collagen (16) and fibronectin (1, 25) have been partially localized, however the chemotactically active regions of laminin and elastin have not yet been established. The present study was undertaken to identify the site(s) of chemotactic activity on elastin.

In approaching the molecular basis of the chemotactic activity of elastin peptides two points seemed important: first, the presence of fibroblast chemotactic activity in tropoelastin (23), the soluble precursor of insoluble elastin (20), suggested that the lysine-derived cross-links characteristic of insoluble elastin are not essential for the chemotactic activity of elastin peptides; and second, the presence of repeating peptide sequences in tropoelastin (4, 19) directed us to look at these peptide repeats as the possible source of the chemotactic activity. In this report, we present evidence that some of the chemotactic activity of elastin is associated with Val-Gly-Val-Ala-Pro-Gly (VGVAPG),[1] a hexamer that repeats six times in one tryptic fragment of porcine tropoelastin (20).

MATERIALS AND METHODS

Preparation of Elastin-derived Peptides: Bovine ligament elastin, obtained from Elastin Products (St. Louis, MO), was solubilized with porcine pancreatic elastase (Sigma Chemical Co., St. Louis, MO) by incubation at a substrate to enzyme ratio of 1:100 (wt/wt) for 24 h at 37°C. The elastase was removed from the elastin digest by affinity chromatography as previously described (23) and the resultant elastin peptides were lyophilized. The concentration of elastin peptides in solution was based upon the dry weight.

[1] *Abbreviations used in this paper:* Boc, tert-butyloxycarbonyl; Bzl, benzyl ester; C5fr, the chemotactic fragments of human C5; EtOAc, ethyl acetate; FMLP, formyl-methionyl-leucyl-phenylalanine; m.p., melting point; PDGF, platelet-derived growth factor; Pet, petroleum; VGVAPG, valyl-glycyl-valyl-alanyl-prolyl-glycine.

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Exhibit A

Synthesis of VGVAPG: Amino acid derivatives were obtained from Bachem Inc. (Torrance, CA) and were of the L-configuration, except for glycine. Isobutyl chloroformate, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride, and 1-hydroxybenzotriazole were purchased from Aldrich Chemical Co. (Milwaukee, WI). Melting points were determined with a Thomas Hoover apparatus and are uncorrected. Thin layer chromatography (TLC) was performed on Whatman silica gel plates (Whatman, Inc., Clifton, NJ) with the following solvent systems: chloroform (C), methanol (M), acetic acid (A); relative flow rate (R_f), CM (5:1); R_f, CMA (95:5:3); R_f, CMA (85:15:3); R_f, CMA (75:25:3); R_f, butanol:acetic acid:water:ethyl acetate (1:1:1:1). The purity of the peptides was also confirmed by carbon-13 nuclear magnetic resonance spectra obtained on a JEOL PFT 100 pulse spectrometer operating at 25.15 MHz with proton noise spin decoupling and an internal deuterium lock. Elemental analyses were performed by MicAnal (Tucson, AZ).

(*a*) Boc-Val-Gly-OBzl: To tert-butyloxycarbonyl-valine (Boc-Val) (8.69 g, 0.04 mol) in acetonitrile (100 ml) cooled to 0°C was added *N*-methyl morpholine (4.4 ml, 0.04 mol). The solution was cooled to −15°C and isobutylchloroformate (5.24 ml, 0.04 mol) was added slowly under stirring while maintaining the temperature at −15 ± 1°C. After stirring the reaction mixture for 15 min. at this temperature, a pre-cooled (−10°C) solution of glycine-benzyl-ester p-tosylate (13.5 g, 0.04 mol) and *N*-methyl morpholine (4.4 ml, 0.04 mol) in dimethyl formamide, 35 ml, was added and stirred overnight at room temperature. Acetonitrile was removed under reduced pressure and the residual dimethyl formamide solution was poured into a cold 4% $NaHCO_3$ solution and stirred for 30 min. The precipitate obtained was filtered, washed sequentially with water, 20% citric acid solution, and water and dried to obtain 13.5 g of the product (yield: 92.6%). A sample was crystallized from ethyl acetate (EtOAc)/petroleum (Pet) ether to give a product with a melting point (m.p.) 80–82°C; TLC: R_f 0.86; R_f 0.53; R_f 0.93. The analysis calculated for $C_{19}H_{28}N_2O_5$ 1/2H_2O was C, 62.44%; H, 7.80%; N, 7.65%. The observed composition was C, 62.62%; H, 7.74%; N, 7.69%.

(*b*) Boc-Val-Gly-OH: Boc-Val-Gly-OBzl (5 g, 13.72 mmol) in glacial acetic acid (50 ml) was hydrogenated at 40 psi in the presence of 10% palladium-carbon catalyst (0.5 g for 6 h. The catalyst was filtered and the filtrate concentrated under reduced pressure. The residue was taken in 4% $NaHCO_3$ solution and extracted with EtOAc (three times). The aqueous solution was cooled, acidified to pH 2 and the peptide extracted into $CHCl_3$ after saturating with NaCl. The combined $CHCl_3$ extracts were washed once with saturated NaCl solution, dried over anhydrous $MgSO_4$ and the solvent removed under reduced pressure. The peptide was precipitated from EtOAc/Pet ether, 3.83 g (Yield: 100%); m.p. 109–113°C; TLC: R_f 0.25; R_f 0.51, R_f 0.7. The analysis calculated for $C_{12}H_{22}N_2O_5$ was C, 52.54%; H, 8.08%; N, 10.21%. The observed composition was C, 52.34%; H, 8.22%; N, 10.28%.

(*c*) Boc-Val-Gly-Val-Ala-Pro-Gly-OCH_3: Boc VAPG-OCH_3 (14) (6 g, 12.89 mmol) was treated with trifluoroacetic acid, 60 ml, for 1 h and the trifluoroacetic acid removed under reduced pressure. The residue was triturated with ether:Pet ether (1:1), decanted, and the oily residue was dried over P_2O_5 and NaOH in a vacuum desiccator. To II (2.92 g, 10.65 mmol) and 1-hydroxybenzotriazole (1.79 g, 11.715 mmol) in dimethyl formamide (25 ml) cooled to −15°C was added 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (2.25 g, 11.715 mmol) and stirred for 15 min. To this was added a pre-cooled (−10°C) solution of the above trifluoroacetic acid salt of the peptide (5 g, 10.65 mmol) in dimethyl formamide (25 ml). The reaction mixture was allowed to come to room temperature and stirred over night. Solvent was removed under reduced pressure and the residue taken in $CHCl_3$ and extracted with water, 4% $NaHCO_3$, water, 10% citric acid, water, dried over anhyd. $MgSO_4$ and the solvent removed. The peptide was crystallized from EtOAc/Pet ether, 3.08 g (yield: 47.2%), m.p. shrinks at 97°C and completely melts at 118°C; TLC: R_f 0.61; R_f 0.3; R_f 0.8. Analysis calculated for $C_{28}H_{48}N_6O_9$ H_2O was C, 53.31%; H, 7.99%; N, 13.32%. The observed analysis was C, 53.40%; H, 7.81%; N, 13.27%.

(*d*) Boc-Val-Gly-Val-Ala-Pro-Gly-OH: Boc-Val-Gly-Val-Ala-Pro-Gly-OCH_3 (1 g, 1.62 mmol) in methanol (10 ml) was stirred with 1 N NaOH (2.02 ml, 2.023 mmol) for 1 h and methanol removed under reduced pressure. The residue was diluted with water and extracted with EtOAc. The aqueous solution was cooled, acidified to pH 2, saturated with NaCl and extracted with $CHCl_3$ (three times). The combined $CHCl_3$ extracts were washed with saturated NaCl, dried over anhydrous $MgSO_4$, and the solvent removed under reduced pressure. The peptide was crystallized from EtOAc/Pet ether, 0.95 g (yield: 98%), m.p. 125–130°C (decomp); TLC: R_f 0.36; R_f 0.56. Analysis calculated for $C_{27}H_{46}N_6O_9$ was C, 54.16; H, 7.74%; N, 14.03%. The observed analysis was C, 54.2%; H, 8.25%; N, 14.17%.

(*e*) HCO_2H·H-Val-Gly-Val-Ala-Pro-Gly-OH: A solution of Boc-Val-Gly-Val-Ala-Pro-Gly-OH (0.6 g, 1 mmol) in 95–97% formic acid (16.5 ml) was stirred for 2.5 h and formic acid removed under reduced pressure. The residue was dissolved in water and lyophilized to obtain the title compound in quantitative yield. TLC: R_f 0.55.

Chemotactic Factors: Platelet-derived growth factor (PDGF), purified from outdated platelet packs (3), was kindly provided by Dr. Thomas F. Deuel, (Jewish Hospital at Washington University Medical Center, St. Louis, MO) and C5-derived chemotactic activity from human serum (C5fr) was a gift from Dr. Donald L. Kreutzer (University of Connecticut Health Center, Farmington). Formyl-methionyl-leucyl-phenylalanine (FMLP) was purchased from Sigma Chemical Corp. (St. Louis, MO).

Antielastin IgG: IgG was prepared from normal rabbit serum and from rabbit antiserum to bovine ligamentum nuchae alpha-elastin by column chromatography using DEAE Affigel-Blue (Bio-Rad Laboratories, Richmond, CA) as described (12). The specificity of the antiserum for elastin has been demonstrated previously (9, 10).

Cells: Fetal bovine ligament nuchae fibroblasts were obtained and cultured as previously described (11). Human mononuclear peripheral blood cells were separated from blood, donated by healthy volunteers, using Ficoll-Hypaque gradients (2). Undifferentiated ligament fibroblasts were induced to synthesize elastin by contact with extracellular matrix material from the ligament of a late gestation calf using procedures recently presented (13).

Assays of Chemotaxis: Chemotaxis was assayed by a double micropore membrane system in modified Boyden chambers, by previously described procedures (21, 22). Briefly, the lower compartment of the chamber, containing 240 μl of test material or basal medium, was separated from the upper compartment containing 360 μl of cell suspension (1.2×10^5 fibroblast/ml or 2.5×10^6 mononuclear cells/ml), by either a 8 μm (fibroblast) or a 5 μm (monocyte) polycarbonate membrane (Nucleopore Corp., Pleasanton, CA) which was overlying a 0.45-μm cellulose nitrate membrane (Millipore Corp., Bedford, MA). After the upper and lower compartments were filled, the chambers were incubated for either 2 h (monocytes) or 6 h (fibroblasts) at 37°C in 5% CO_2-balance air. The chambers were then disassembled and the membrane pairs removed and stained with hematoxylin. In each experiment, the data points represent the mean of cell counts of five high power (× 400) fields on each of three membrane pairs. The results are reported as the mean number of cells that migrated through the upper membrane, corrected for blanks as determined by chambers containing only medium in the lower compartment. All experiments included positive controls: for fibroblast studies, PDGF, 30 ng/ml (1 nM), was put in the lower compartment; for monocyte studies, FMLP, 10^{-8} M and/or C5fr was put in the lower compartment.

To assess the capacity of elastin peptides and VGVAPG to desensitize monocytes to the chemotactic activity of elastin peptides and VGVAPG, we incubated mononuclear cells for 30 min at 25°C with either elastin peptides, 100 μg/ml or VGVAPG, 10^{-8} M, respectively, and then washed the cells three times with basal medium. Chemotaxis was then assayed as described above.

The effects of antielastin IgG on chemotaxis to VGVAPG and elastin peptides were determined by preincubating antielastin IgG with either VGVAPG or elastin peptides for 60 min at 37°C and then assaying the mixture for chemotactic activity. In these experiments, controls included (*a*) VGVAPG or elastin peptides preincubated with preimmune IgG and (*b*) mixtures of antielastin IgG preincubated with PDGF.

RESULTS

Chemotactic Activity of VGVAPG

VGVAPG caused fibroblast and monocyte migration, with a maximal activity in the range of 10^{-9}–10^{-8} M (Fig. 1). At

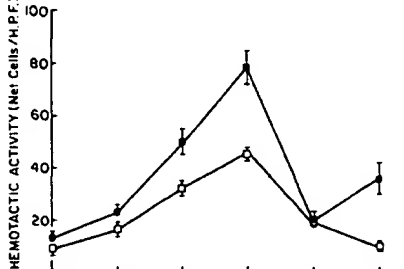

FIGURE 1 Fibroblast and monocyte migration in response to VGVAPG. Mean and standard error are shown, $n = 15$. Background cell migration was 16 for the fibroblast (□) studies and 36 for the monocyte (■) experiments.

TABLE I

Fibroblast Chemotaxis to VGVAPG

VGVAPG (M), lower compartment	VGVAPG (M), upper compartment			
	0	10^{-10}	10^{-9}	10^{-8}
0	(7)	1 ± 0.6	0 ± 0.7	−2 ± 0.5
10^{-10}	4 ± 1.0*	2 ± 0.7	−3 ± 0.4	1 ± 1.0
10^{-9}	15 ± 1.7	7 ± 1.3	0 ± 0.4	0 ± 0.6
10^{-8}	27 ± 1.7	13 ± 1.8	3 ± 0.6	1 ± 0.7

* Net cells per high power field ± SEM, n = 15. Positive control PDGF, 10^{-9} M = 31 cells per high power field.

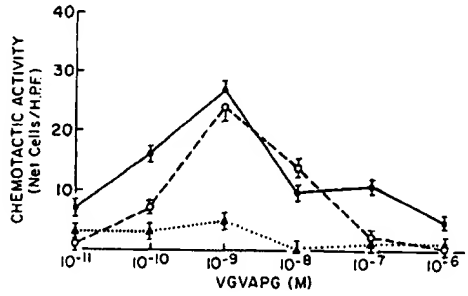

FIGURE 2 The effect of antielastin IgG on the fibroblast chemotactic activity of VGVAPG. In these studies background cell migration was 11. (●) No IgG. (○) Pre-immune IgG. (▲) Antielastin IgG. The error bars are as in Fig. 1.

the peak of activity, the number of cells that migrated was nearly as great as that observed with the optimal PDGF concentration for fibroblast chemotaxis, 10^{-9} M (21), and the optimal concentration of FMLP for monocyte chemotaxis, 10^{-8} M. By "checker board analysis" the fibroblast migration to VAVAPG was chemotactic, not chemokinetic (Table I).

Effect of Antielastin IgG upon the Chemotactic Activity of VGVAPG

Polyclonal antielastin IgG, previously shown to inhibit the chemotactic activity of elastin peptides for monocytes (22), abolished the chemotactic activity of VGVAPG (Fig. 2). Specificity of the inhibition was shown by demonstrating that antielastin IgG had no inhibitory effect against the chemotactic activity of PDGF and that pre-immune rabbit IgG exerted no inhibition upon the chemotactic activity of VGVAPG. Moreover, immune complexes consisting of VGVAPG plus antielastin IgG did not block the chemotactic activity of FMLP, indicating that these immune complexes do not have a generalized inhibitory effect upon chemotaxis.

Desensitization to VGVAPG

Preexposure of monocytes to elastin peptides before testing them for chemotaxis completely abolished their chemotactic responses to both elastin peptides and VGVAPG (Fig. 3*A*). The loss of chemotactic responsiveness was specific since the cells still migrated normally to two other unrelated chemotactic factors, C5fr and FMLP. Preincubation with VGVAPG completely blocked chemotaxis to VGVAPG but only partially reduced chemotactic responses to elastin peptides (Fig. 3*B*), suggesting that elastin peptides contain chemotactically active sequences other than VGVAPG.

Chemotactic Responses of Undifferentiated Ligament Fibroblasts to VGVAPG

Undifferentiated ligament fibroblasts showed no migration in response either to VGVAPG or elastin peptides, but were fully responsive to PDGF (Fig. 4, *top*). After these cells were exposed to extracellular matrix from 270-d gestation bovine ligamentum nuchae, however, they responded to both VGVAPG and elastin peptides (Fig. 4, *bottom*).

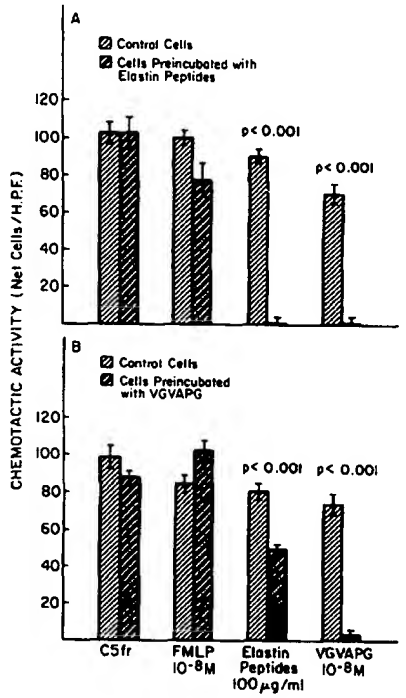

FIGURE 3 Monocyte chemotaxis to C5fr, FMLP, elastin peptides, and VGVAPG after preincubating the cells with either (*A*) elastin peptides or (*B*) VGVAPG. The background numbers of migrating cells for these experiments were 49 and 51, respectively. The error bars are as in Fig. 1.

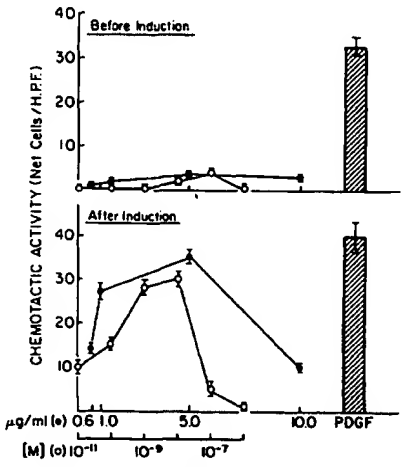

FIGURE 4 Chemotaxis of undifferentiated ligament fibroblasts to VGVAPG and elastin peptides before and after the cells were induced to synthesize elastin by exposing them to matrix from differentiated ligament fibroblasts. (*Top*) Before induction. (*Bottom*) After induction. Background cell migration for these studies was 19 and 25, respectively. (●) Elastin peptides. (○) VGVAPG. The error bars are as in Fig. 1. The PDGF standards were 30 ng/ml, 10^{-9} M.

DISCUSSION

Working with tryptic fragments of tropoelastin extracted from the aortas of copper-deficient pigs, Sandberg et al. (20) have succeeded in sequencing nearly all of this molecule of approximately 800 amino acid residues. The tryptic fragments of tropoelastin appear to be divisible into two groups: (*a*) small peptides of two to four amino acids, consisting mainly of alanine and lysine, and thus parts of the molecule destined to be involved in cross-links; and (*b*) more hydrophobic peptides of 17–81 amino acids, some of which contain repeating sequences (19). The principal repeating sequences are a tetrapeptide, GGVP, occurring several times in a peptide near the N-terminus, a pentapeptide, PGVGV, which repeats 11.4 times in one peptide of 81 residues, and a hexamer, VGVAPG, used in the present study, which repeats six times within a stretch of 57 amino acids. The functions served by these repeats are not yet clear, but it has been postulated that they have a specific role in the physical properties of elastin (28, 29). In addition to having chemotactic activity it is clear that the hexameric repeat is a strong antigenic epitope (10), suggesting that this region of the molecule may be an important biologically active domain of elastin.

It should be emphasized that the present work was limited to the repeating hexamer. The results therefore do not exclude the presence of chemotactic activity associated with other parts of tropoelastin or elastin peptides, especially other repeats. Indeed, the observations that the chemotactic activity to elastin peptides is only partially blocked by preexposure of the target cells to VGVAPG, strongly suggests that elastin peptides have chemotactically active sites besides VGVAPG sequences. Moreover, we have not yet determined whether all six residues are necessary for the activity of the hexamer nor whether the order of the residues is critical. It should also be noted that while there are no sequence data on bovine elastin, the source of the elastin peptides used in the present work, studies of the amino acid composition and of the immunoreactivity of elastin suggest conservation of the sequence between species (9, 10, 26).

The finding that a small hydrophobic repeating peptide in elastin is chemotactic for fibroblasts and monocytes is analogous to observations of chemotaxis to collagens, reported by Gauss-Muller et al. (5) and by Postlethwaite (16). Interstitial collagens, types I, II, and III, and basement membrane collagens types IV and V are chemotactic for fibroblasts and monocytes and the activity is retained even if the collagens are subjected to proteolytic cleavage with mammalian and bacterial collagenases or with cyanogen bromide. The chemotactic activity of isolated collagen chains matches their capacity to bind to fibroblasts and the capacity of collagen fragments to inhibit the binding of collagen chains to fibroblasts correlates with their chemotactic activity. Of particular relevance to the present report, synthetic di- and tri-peptides from collagen containing hydroxyproline also have fibroblast chemotactic activity, suggesting that cellular recognition of collagens may involve very short amino acid sequences. The cell attachment activity of fibronectin has been localized to within a 30-amino acid peptide (15). Since the chemotactic activity of fibronectin appears to be located near or coincident with the cell attachment region of the molecule (1, 7, 25), fibronectin may be another example of an extracellular matrix macromolecule with chemotactic activity that will ultimately be localized to a small peptide segment.

Hunninghake et al. (6) first demonstrated that monocytes remained chemotactically responsive to elastin peptides after exposure to activated serum, suggesting that recognition of elastin peptides by monocytes does not involve the C5a receptor. The present study confirms and extends this observation: (*a*) elastin peptides are shown not to utilize the FMLP receptors on monocytes (30); and (*b*) a peptide not previously studied for monocyte chemotaxis (VGVAPG) has been identified as a monocyte chemoattractant and to use neither the C5a or the FMLP receptors. It is also worthy of emphasis that being able to demonstrate that a synthetic analog of a repeating peptide in elastin is chemotactic for fibroblasts and monocytes strengthens the earlier reports that elastin-derived peptides are chemotactic (6, 22, 23) because it excludes the possibility that the previously reported activity was due to a contaminating substance(s) in proteolysed elastin preparations.

The physiological roles that chemotaxis to tropoelastin and elastin peptides might serve in vivo can only be speculated on at this time. Recognition by monocytes might facilitate removal of proteolysed elastin. For fibroblasts, chemotaxis to elastin peptides might serve to promote wound healing and matrix remodeling generally, however, there may be purposes for fibroblast recognition of elastin peptides and tropoelastin besides cell movement. Recognition, which we have measured in vitro as chemotaxis, might have a role in cell activities such as regulation of elastin synthesis or orientation of tropoelastin molecules on the cell surface to facilitate cross-linking.

Apart from helping to identify the sites of chemotactic activity on elastin, the results of the present study represent another illustration of changes in chemotactic responsiveness with development. We have shown elsewhere that undifferentiated ligament fibroblasts do not show chemotactic responsiveness to elastin peptides until after induction of differentiation as reflected by the onset of elastin synthesis (8). Here, we have presented a similar picture with respect to a repeating hexamer of elastin.

These results indicating that VGVAPG is an important component of the chemotactic activity of elastin peptides suggest that the hexamer will be a useful probe to explore interactions between cells and tropoelastin and elastin peptides such as binding reactions and metabolic effects. The results also indicate that the preparation of artificial elastomers using peptide repeats of tropoelastin (28, 29) may lead to materials that have biologically interesting properties as well as the physical characteristics of native elastin.

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Note Added in Proof: Recent studies indicate that tropoelastin does not completely desensitize monocytes to the chemotactic activity of elastin-derived peptides. Thus, domains unique to insoluble elastin contribute to the overall chemotactic activity of peptides generated from the insoluble molecule.

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